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PHYSIOLOGICAL AND PHARMACOLOGICAL MANIPULATIONS WITH LIGHT FLASHES

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INTRODUCTION

In the experiments described here, a physiological measurement is made while photochemical procedures are employed to alter (*a*) the concentration of a ligand near membranes or proteins or (*b*) the structure of the ligand-receptor complexes. Because photochemical reactions often provide the quickest way to produce such chemical perturbations, we emphasize the kinetic information that such experiments have yielded. This information requires a suitably rapid physiological measurement, usually an electrical or optical one. The results often complement those obtained with other kinds of kinetic investigation (iontophoretic application of drugs, stopped-flow mixing, temperature jump, etc). Pharmacological manipulations with light flashes are especially useful for biological systems that cannot be flowed, for instance membranes under electrophysiological investigation or solutions at very low temperatures.

In a conceptual sense, this chapter could treat flash-photolysis investigations of heme proteins; but the topic has recently been reviewed authoritatively (84). This review does not concern photoaffinity labeling (28) or studies on fluorescence recovery after photobleaching.

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PHOTOCHEMICAL STRATEGIES

General Constraints

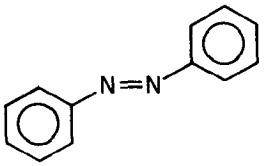
For pharmacological experiments with light flashes, one requires a photosensitive molecule that satisfies several criteria.

1. The compound must be soluble in aqueous solutions of moderately high ionic strength.
2. The photochemical reaction must also proceed in this medium.
3. The photochemical reaction should take place instantaneously with respect to the time scale (usually milliseconds to seconds) of the physiological phenomenon being studied, without the formation of reactive intermediates or active by-products.
4. The photoproduct should be stable (thermally and solvolytically) on the same time scale so that the flash produces a true "concentration jump" of active molecules. (On the other hand, for some studies it may be quite useful to generate a "concentration pulse" if the time course of the pulse is accurately known.)
5. The photochemical reaction should proceed at wavelengths long enough to cause no significant damage to cellular components. A tentative guideline for this criterion would be $\lambda > 300$ nm, although an efficient photochemical strategy might produce acceptably low damage for wavelengths down to about 250 nm.
6. The efficiency per incident photon should place the reaction within reach of flashlamps and collimated (rather than focused) pulsed lasers. A tentative guideline for this requirement is that the extinction coefficient, ε, times the quantum yield, ϕ, for the reaction should exceed about 500.
7. Both the precursor and the photoproduct should have simple, well-characterized equilibrium effects on the physiological system; there should be no complicating interactions with proteins or membranes.

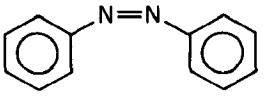
These combined constraints are known to be satisfied by only a few of the many available photochemical processes and photosensitive molecules.

Photoisomerization

CIS-TRANS PHOTOISOMERIZATION OF AZOBENZENES Azobenzene and many of its derivatives can exist in both *cis* and *trans* configurations with respect to the axis of the azo nitrogens.



trans



cis

The *trans* configuration is planar; but in the *cis* configuration, steric hindrance between the two *ortho* hydrogens forces the benzene rings into two planes skewed at 53° (4). As a result, the two configurations differ in many physical properties such as thermochemistry, dipole moment, and absorption spectra (92). It is not surprising that certain azobenzene derivatives display different pharmacological properties in the two isomeric configurations, as described in more detail below.

Some photoisomerizable azobenzene derivatives have photophysical properties well studied for kinetic experiments on membranes and proteins. The *cis* → *trans* and *trans* → *cis* photoisomerizations are produced by light of wavelengths 410–450 nm and 300–350 nm, respectively. The photoisomerizations occur within a microsecond after absorption of a photon (94) and with a high quantum yield. Photoisomerization is the only result of photon absorption: there are no long-lived excited states, reactive intermediates, or competing photoreactions. The *cis* and *trans* configurations are thermally stable for at least several seconds and, with most of the molecules, for several days. These photoisomerizable compounds have the drawback that flashes lead to a photostationary mixture of the *cis* and *trans* configurations, rather than to pure solutions of one or the other configuration. However, the composition of the photostationary state and the rate of approach can be determined experimentally. For a given compound, the composition of the photostationary state depends on the wave-length of irradiation, λ. If the *cis* and *trans* isomers have molar absorption coefficients $\varepsilon_{c,t}(\lambda)$ respectively, and quantum yields $\phi_{c,t}(\lambda)$ for photoisomerization, then the mole fraction of *cis* isomers in the photostationary state is $\varepsilon_t\phi_t/(\varepsilon_t\phi_t + \varepsilon_c\phi_c)$. For the molecule we have studied in most detail, Bis-Q, the highest percentage of *cis*, 94–96%, is produced by 338–341 nm light (35, 79); and 420–440 nm light produces 66% *trans* isomers. In dilute solutions, the photostationary state is approached exponentially with the number of flashes; the intensity $I(\lambda, t')$ of the flash (where t' is time) determines the "rate" constant $K_c + K_t$ (in units of flash^{-1}), where $K_{c,t} = \int_\lambda \int_{t'} I(\lambda, t')\varepsilon_{c,t}(\lambda)\phi_{c,t}(\lambda)\,dt'\,d\lambda$. For a weak flash of intensity $dK_{c,t}$, the fraction of molecules that undergo photoisomerization is $dK_{c,t}$. Further-

more, the percentage of *cis* in the photostationary state is given by $K_t/(K_c + K_t)$. The parameters $K_{c,t}$ can be determined, for a particular flash intensity and a particular compound, by spectral measurements during the approach to the photostationary state. Thus it is not necessary to know I, ε, and ϕ individually. With flashlamps and pulsed lasers, values of about one flash^{-1} can now be achieved for both K_c and K_t (65, 79, 81), so that a single flash converts the solution most of the way to the photostationary state.

The photoisomerization properties are not expected to be sensitive to any solvent or binding conditions likely to occur in or near biological membranes (19, 27, 118). These expectations have recently been partially verified with spectral measurements on azobenzene molecules that were covalently bound to bovine serum albumin (95) or to detergent-solubilized membrane proteins (94). Laser flashes photoisomerized these bound molecules with $K_{c,t}$ values identical to those found with the unbound molecules in aqueous solution.

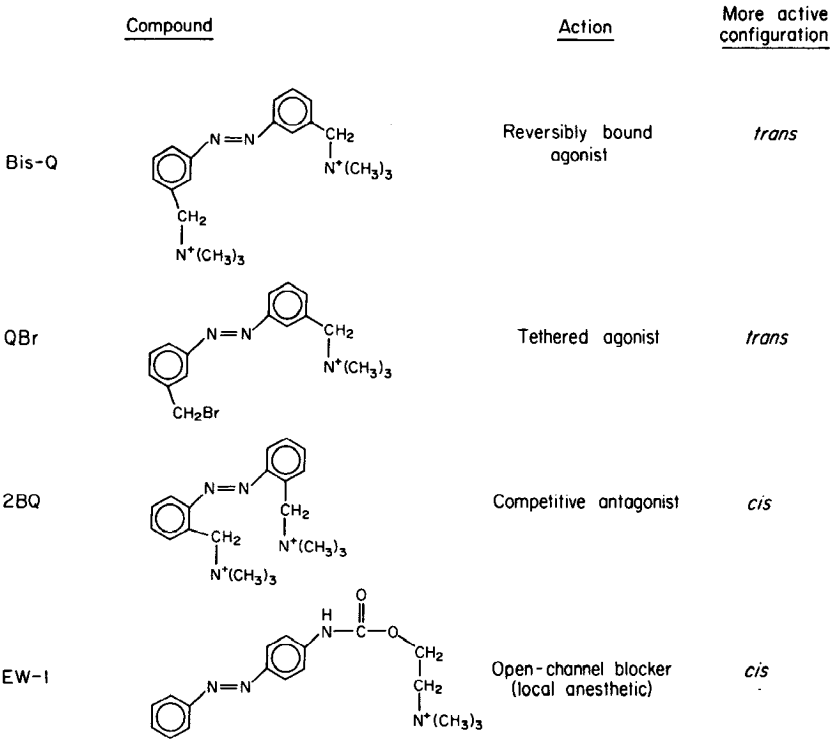


Figure 1 Photoisomerizable cholinergic compounds designed and synthesized by N. H. Wassermann and B. F. Erlanger.

In summary, the azobenzene derivatives in Figure 1 fulfill the requirements for calibrated "concentration jumps" that are instantaneous on the time scale of channel gating in biological membranes.

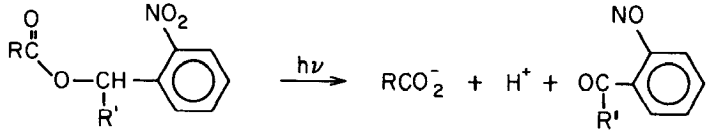
Photoisomerizable azobenzene groups have been incorporated into crown ethers. The two isomers extract alkali metal cations into liquid membranes to differing extents (96). Such molecules also might provide a means for controlling the permeability of biological membranes.

OTHER PHOTOISOMERIZABLE COMPOUNDS Some 2,2′ stilbene disulfonates block the anion exchange system of erythrocyte membranes (15, 23); and the *cis* and *trans* isomers have different potencies (39, 40, 97). No measurements have been reported on modifications to anion flux during photoisomerization of these compounds.

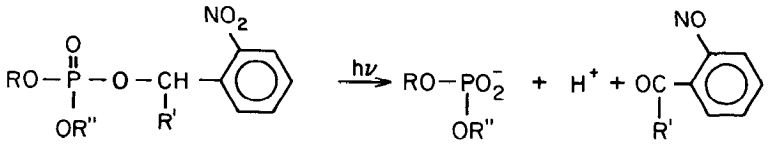
Cyanine, merocyanine, carbocyanine, and related dyes undergo several types of photochemical reaction. In the best-studied photoisomerization reaction, absorption of a photon by spiropyrans leads to C-O bond cleavage, producing a merocyanine (102). Other possible photoreactions include oxidation-reduction, excimer, and long-lived triplet formation. The resulting charge rearrangements lead to photovoltages in lipid bilayer membranes exposed to certain dyes (11, 35, 50, 51, 106, 107). Experiments are also underway to employ these or similar effects to photostimulate living cells (44; A. Grinvald, personal communication).

Photochemical Cleavage of Blocking Groups

Several small aromatic groups can be cleaved with light of wavelength greater than 300 nm in aqueous solution (reviewed in 87). The most common scheme exploits the light-induced internal oxidation-reduction reaction in nitroaromatics with a benzylic hydrogen *ortho* to the nitro group (7, 78). The *o*-nitrobenzyl moiety has been removed photochemically from esters to form carboxylate groups (12, 86).

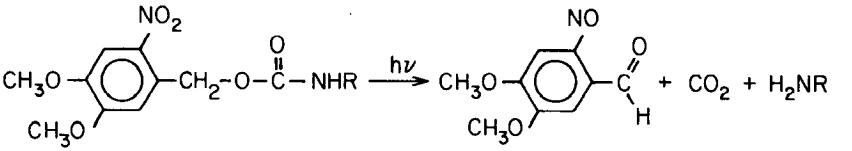


The phosphodiester or phosphotriester is similarly cleaved to form phosphates (56).



The phosphodiester photolysis has been studied kinetically, as described below; under some circumstances the phosphate is formed within milliseconds (75). In the simplest *o*-nitrobenzyl derivative, R′=H, and the photolysis results in a nitroso aldehyde byproduct (36, 37). This aldehyde apparently causes few problems at micromolar concentrations (37); but at higher concentrations it participates in further reactions that vitiate the compounds' usefulness. These problems can be avoided by derivatizing the benzylic carbon, so that the less reactive ketone is formed instead. Thus the *o*-nitrobenzyl group has been photoremoved from compounds in which R′ is a phenyl (12), methyl (53), or another *o*-nitrobenzyl group (86).

In extensions of this technique, 2-nitrobenzyloxycarbonyl and 6-nitroveratryloxycarbonyl derivatives of amino groups can be photolyzed to yield the free amino groups (8, 12, 86).



Because many neurotransmitters and local anesthetics contain amino groups critical to their function, this reaction is of great potential interest. *o*-Nitrobenzyl ethers have also been prepared and photolyzed to yield hydroxyl groups in phenols (6), sugars (8, 116, 117), and nucleotides (14, 22, 83, 84). Both the amino and hydroxy groups can be photogenerated in aqueous solution, but nothing is known about the speed of the reactions.

The 5-bromo-7-nitroindolinyl moiety is another promising photocleavable group, particularly because photosolvolysis proceeds with light of wavelength 420 nm (as compared with 350 nm for the *o*-nitrobenzyl group) (5, 43).

SYNAPTIC TRANSMITTERS

Studies on Nicotinic Acetylcholine Receptors

ELECTROPHYSIOLOGY Pharmacological manipulations with light flashes complement several powerful electrophysiological methods for kinetic studies on ion channel gating by nicotinic acetylcholine receptors [most recently reviewed in (2)]. In all modern studies, the voltage-clamp technique is applied either to whole cells or to microscopic ($\sim 1\ \mu m^2$) patches of membrane (44a). Voltage-clamp currents are proportional to the

number of open channels in the membrane, with a temporal resolution of several tens of microseconds in favorable cases, as the channel population responds to forced or spontaneous perturbations. The available perturbations may be summarized as follows.

1. The neurally evoked postsynaptic current is produced by an impulse that propagates into the presynaptic nerve terminal. A brief but concentrated pulse of acetylcholine is liberated into the synaptic cleft between the presynaptic and postsynaptic cells. Acetylcholine binds to receptors; channels open rapidly so that the current reaches a peak within ~ 200 μs; and the transmitter is then efficiently hydrolyzed by acetylcholinesterase as it dissociates from receptors. As a result, very few channels open after the peak. Therefore the neurally evoked postsynaptic current declines exponentially with a rate constant equal to that for channel closing (2, 72, 73, 111).

2. The voltage-jump relaxation is performed in the presence of a constant, externally applied agonist concentration. This experiment exploits the voltage sensitivity of the agonist-receptor interaction, so that the voltage-clamp circuit is employed both to produce the perturbation and to measure its consequences. Voltage-jump experiments give information about both opening and closing rates.

3. In fluctuation analysis, all the stimulating parameters are kept constant and one studies the fluctuations in voltage-clamp currents produced by spontaneous deviations about equilibrium.

4. In favorable circumstances, one can resolve the step-like changes associated with the opening and closing of individual channels (44a).

PHOTOCHEMISTRY Based on earlier studies with blockers of serine esterases (16–18, 38, 54, 109), Erlanger and Wassermann designed and synthesized a series of azobenzene derivatives that interact with nicotinic acetylcholine receptors (Figure 1). These molecules have two crucial properties: (*a*) they are photoisomerizable, as described above; (*b*) the two configurations differ in their pharmacological properties.

THE AGONIST, BIS-Q At the nerve-muscle and nerve-electroplaque synapses of several South American and African fishes, *trans*-Bis-Q acts as a potent but typical nicotinic agonist at concentrations on the order of 10^{-7} M (13, 63, 65, 81, 113). At 10°C and a membrane potential of -75 mV, it interacts with acetylcholine receptors to open channels with a conductance of 26 ps and a lifetime of 4 msec;[1] both these values are

[1]Known nicotinic agonists induce channels with roughly a tenfold range of durations when tested under identical conditions. The actual duration depends multiplicatively on several conditions, such as temperature, membrane voltage, and synaptic vs extrasynaptic location. *Trans*-Bis-Q and acetylcholine induce channels of relatively long duration.

identical to those measured with acetylcholine itself under the same conditions (113). The rate constant for opening increases with *trans*-Bis-Q concentration (65, 81). The dose-response curve is sigmoid, with a Hill slope very near 2 (93). The agonist-induced conductance can be blocked by the competitive antagonist, tubocurarine (63, 65), and by the "open-channel blocker", QX-222 (65), and desensitizes with continued exposure to Bis-Q.

Electroplaques from the giant Amazonian electric eel, *Electrophorus electricus*, constitute a particularly suitable preparation for combined electrophysiological and photochemical experiments on Bis-Q. The *cis* configuration is less than 100 times as potent an agonist as the *trans* configuration; indeed, any channels induced by solutions of nominally pure *cis* isomer could be due to trace contamination by *trans*-Bis-Q. At concentrations of 1 μM or less, *cis*-Bis-Q also shows no inhibitory action, either as a competitive antagonist or as an "open-channel blocker." Thus *cis*-Bis-Q apparently fails to bind to acetylcholine receptors. This situation made it possible to generate information from two unique types of photochemical perturbation.

The first experiment is the photochemically-induced "concentration-jump" of agonist. One begins with a solution of *cis*-Bis-Q and uses a flash lamp or, more recently, a pulsed laser to produce a sudden step of *trans*-Bis-Q concentration. A series of such experiments (59, 61, 67) culminated in the observation that receptor channels begin to open within 10 μs after the first agonist molecules appear near receptors. This datum reduced (by about tenfold) the upper limit on the delay that the agonist-receptor interaction contributes to the transmission of a nerve impulse across a nicotinic synapse. It was hoped that the detailed time course of such "concentration-jump relaxations" would yield new data on agonist-receptor binding, conformational changes, or other events leading to channel activation. An initial delay, in particular, could have been of interest. However, thus far the concentration-jump relaxations have been single exponential functions of time (63, 65, 81), like voltage-jump relaxations and autocorrelation functions measured with Bis-Q and other agonists. The exponential time course is maintained even at the earliest times after the flash, within the resolution of the voltage-clamp circuitry (50–100 μs) (M. S. Brodwick, R. E. Sheridan, and H. A. Lester, unpublished).

A second type of photochemical perturbation with Bis-Q has, however, produced uniquely interesting information. In this experiment, a flash produces *trans* → *cis* photoisomerization of *trans*-Bis-Q molecules that are bound to receptors with open channels. This photochemical perturbation does not depend on a "concentration jump" of agonist near receptors; it

is instead a "molecular rearrangement" of the agonist-receptor complex itself. Thus the experiment is analogous to flash-photolysis experiments on liganded hemoproteins, with the difference that subsequent events are monitored with electrophysiological rather than with optical techniques. As a consequence of the *trans* → *cis* photoisomerization, receptors are no longer occupied by agonist molecules; therefore channels close. The resulting rate of channel closing is one to two orders of magnitude faster than the rate of channel opening produced by the "concentration-jump" described in the preceding paragraph (61, 81) and is, in fact, the fastest known kinetic process at acetylcholine receptors. This signal has been termed phase 1; and its speed allows its measurement uncomplicated by slower processes.

Phase 1 has been analyzed for information on the temporal and stoichiometric aspects of coupling between agonist binding and channel activation (81, 93, 94). The theory is that each receptor's channel remains open if and only if n agonist molecules are present at the receptor's binding site. Thus the channel closes if any of the bound *trans*-Bis-Q molecules are photoisomerized to the *cis* configuration. The photon cross section for channel closing is therefore n times that for *trans* → *cis* photoisomerization. The experiments involved comparing the effects of flashes on (a) the fraction of Bis-Q molecules that undergo *trans* → *cis* photomerization (K_t, measured spectrally) and (b) the fraction of channels that close (measured electrophysiologically as the amplitude of phase 1). The ratio was $n = 2.06 \pm 0.09$ (mean ± SEM). This result provides a very direct confirmation of the concept that the open state of a receptor's channel is much more likely to be associated with the presence of two bound agonist molecules than with a single bound agonist molecule. This result also verifies that both receptor molecules are bound the entire time the channel remains open. It can also be argued, albeit less directly, that at least one agonist molecule leaves the receptor as the channel closes (81).

Bis-Q is much less potent at the acetylcholine receptors of many other teleost fishes (M. E. Krouse, M. M. Weinstock, H. A. Lester, unpublished results), of elasmobranch fishes (60), and of reptiles (M. M. Weinstock, unpublished), producing observable conductances at several tens of μM. Kinetic experiments with these less responsive preparations are complicated by the fact that Bis-Q also exerts "open-channel blockade" at such concentrations (3). At frog, rat, and mouse end plates, *trans*-Bis-Q has no detectable potency as an agonist. Several agonists are known (for instance decamethonium) whose potency differs among nicotinic receptors from various animals, but the species variations seen with Bis-Q are probably the largest reported. The reasons are not known. Perhaps the

azo double bond interacts strongly with only some receptors (110); perhaps only some membranes are suitable environments for the *trans*-Bis-Q molecule's combination of large dipole moment accompanied by hydrophobic regions (35).

THE TETHERED AGONIST, QBr Silman & Karlin (98) designed and synthesized agonists that can be covalently linked to the acetylcholine receptor at a sulfhydryl group (formed by previous reduction with dithiothreitol) near the binding site for agonists. These compounds, which have been termed tethered agonists (65), produce a persistent activation of channels. They offer two advantages for kinetic studies. (*a*) Tethered agonists eliminate the first step in the agonist-receptor binding reaction, namely the initial, diffusion-limited encounter with receptors. (*b*) Because the unreacted molecules are washed out of the preparation after the tethering reaction, there is no chance that they can also function as competitive antagonists or "open-channel blockers." Their disadvantage is that, like all known agonists, they eventually desensitize receptors, so that the number of open channels decreases with time (31, 32). This shortcoming is avoided by the photoisomerizable tethered agonist, QBr (13, 65; Figure 1). QBr is tethered to receptors in its active, *trans* configuration and can then be photoisomerized to a predominantly *cis* mixture that neither opens channels nor desensitizes them, until the moment of the voltage-clamp test. This feature, in conjunction with advantage (*b*) above, may render tethered QBr a more generally useful ligand than Bis-Q for studies of nicotinic acetylcholine receptors. In addition to *Electrophorus* electroplaques, QBr has been tethered to the acetylcholine receptor of skate electroplaques, where its properties resemble those seen with *Electrophorus* (H. A. Lester, M. E. Krouse, M. M. Weinstock, unpublished). As expected for a tethered agonist, the QBr-induced conductance cannot be blocked by the competitive antagonist, tubocurarine, but remains sensitive to "open-channel blockers" such as QX-222. Also as expected, the relaxation kinetics reveal that the channel opening rate is governed by an intramolecular transition, rather than by a bimolecular process involving free agonist molecules and membrane-bound receptors (65).

Apart from these expected differences, the channels opened by QBr and other tethered agonists share many properties with those opened by reversibly bound agonists (31, 32, 65). Channels fluctuate between open and closed states. Voltage-jump relaxations follow an exponential time course; their analysis reveals that the rate constant for channel opening does not depend on membrane voltage, whereas the rate constant for closing depends on voltage to about the same extent as for reversibly bound agonists. The actual magnitudes of these rate constants are about

twice as great as those seen with 50 μM carbachol, a typical reversibly bound agonist, and have the same sensitivity to temperature. As with reversibly bound Bis-Q, tethered QBr is an agonist in the *trans* configuration but tethered *cis*-QBr does not open channels. These similarities all suggest that the same rate-limiting step governs the opening and closing of channels for both reversibly bound and tethered agonists. Therefore, this step is probably not the initial, diffusion-limited encounter between agonist and receptor molecules. Instead, the rate-limiting step is some subsequent rearrangement or conformational change of the agonist-receptor-channel complex, as discussed in detail by several authors (2, 42, 65, 72, 73).

With tethered QBr as with reversibly bound Bis-Q, uniquely interesting information comes from experiments in which the agonist-induced conductance is measured while flashes produce molecular rearrangements of the agonist-receptor complex (65). Channels are opened by *cis* → *trans* photoisomerizations and closed by *trans* → *cis* photoisomerizations; this cycle can be repeated many times. The kinetics of the relaxations confirm that one is dealing with intramolecular events, with no dependence on free agonist molecules. The amplitudes of the conductance changes were compared with the known photochemical consequences of each flash. The results were analyzed in terms of models that specified (*a*) whether one or two tethered QBr molecules were associated with each channel and (*b*) whether channel opening required that one or two tethered QBr molecules have the *trans* configuration. Each scheme predicts a different relation between the fraction of molecules in the *trans* configuration and the fraction of active receptors. The conclusion was that each channel's activation is controlled by the configuration of a *single* tethered QBr molecule. This result is surprising in view of the considerable evidence (some of it discussed above) that two *reversibly* bound agonist molecules are required to activate a single channel. It may be asked whether this change in functional stoichiometry is caused (*a*) by the reduction of disulfide bonds in preparation for the tethering reactions, or (*b*) by the covalent attachment of the agonist molecule. Technical difficulties have thus far prevented a direct answer to this question.

THE PHOTOISOMERIZABLE "OPEN-CHANNEL BLOCKER," EW-1 According to present concepts, local anesthetic molecules act by prematurely terminating the open state of ionic channels in nerve membranes (1, 9, 20, 101). Channels open normally, and with normal conductance; but in the open state there is an additional binding site for the blocking molecule. The voltage dependence of blockade for charged blockers suggests that the binding site is within the membrane (2), but it is not yet

known whether this site is (*a*) directly within the channel, so that the local anesthetic would act like a plug in a drain, or (*b*) at an allosteric site elsewhere on the receptor or channel. Also unclear is whether the blocking drug approaches its binding site from the aqueous phase (intracellular for sodium channels and extracellular for acetylcholine receptor channels) or from within the membrane; probably the two routes contribute to varying extents for different blocking molecules.

This model makes the explicit statement that the blocking molecule reaches its binding site only after the channel opens. Thus one predicts that blockade ought to proceed as usual even though the blocking molecule is created after the channel opens. Because the blocking potency of EW-1 (Figure 1) differs in its two configurations, this molecule enabled a test of the prediction (64; Figure 2). The experiment employed the brief pulse of acetylcholine that is released in response to a nerve impulse in the presynaptic terminals. As outlined above, receptors are activated nearly synchronously; acetylcholine is then hydrolyzed rapidly

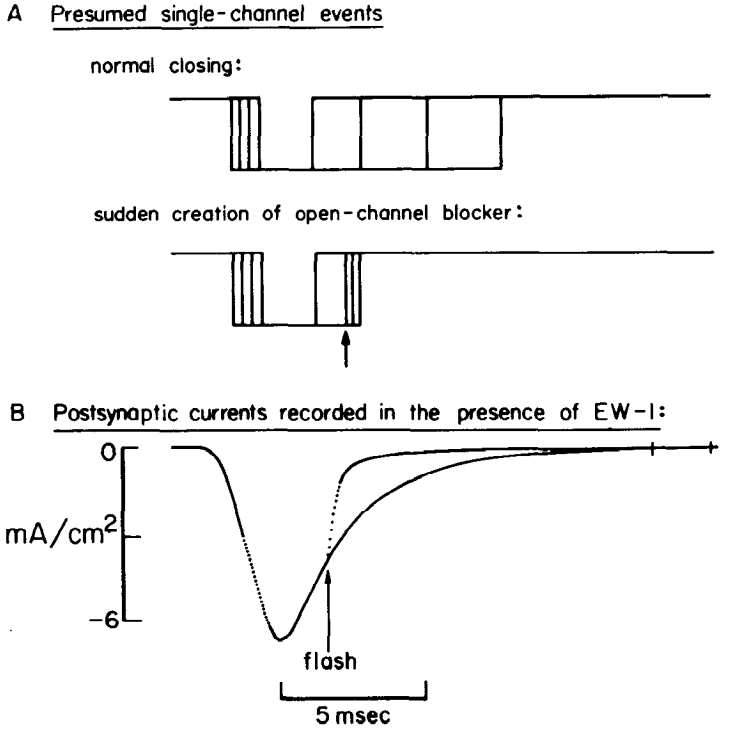


Figure 2 Explanation of an experiment with the light-activated "open-channel blocker", EW-1.

by acetylcholinesterase, and no more channels open. Thus, the decay phase has an exponential time course formed by the stochastic closing of individual receptor channels. During this decay phase, a flash was delivered to create *cis*-EW-1, the blocking configuration. The experiment would be more elegant if it employed single-channel recordings like the imaginary ones of Figure 2*A*; but the macroscopic record of Figure 2*B* plainly shows that the decay phase was accelerated by the flash. Because no channels open during the decay phase, the newly created *cis*-EW-1 molecules must be prematurely terminating open channels, in agreement with the prediction.

Interestingly, the flash-induced blockade in a given *trans*-EW-1 solution is temporarily much greater than the steady-state blockade in a nearly pure *cis*-EW-1 solution of equal concentration (64). Most of the blockade disappears with a time constant of about 300 msec. This transient block depends on the time since the flash rather than on the time since the opening of channels. Therefore the transient block should not be confused with the "open-channel blockade" model's prediction that at low agonist concentrations, an open-channel blocker ought to produce major kinetic changes but no appreciable steady-state blockade (2). Instead, the temporarily increased blockade probably arises because EW-1 molecules (*a*) accumulate at the water-membrane interface with their hydrophobic azobenzene "tails" in the membrane and (*b*) are liberated near channels by a flash (35). Thus EW-1 exhibits an additional nonspecific interaction with membranes, in violation of constraint No. 7. This phenomenon could possibly be exploited for information on the extent of local anesthetic penetration into membranes. Also worthy of further study is the prediction that channels ought to conduct again if *cis*-EW-1 could suddenly be flashed off the binding site.

THE PHOTOISOMERIZABLE COMPETITIVE ANTAGONIST, 2BQ Tubocurarine, the classical competitive antagonist, is thought to block receptors by binding to the agonist site, preventing normal activation.[2] Little is known about the kinetics of antagonist-receptor binding, however. The pioneering experiments with iontophoretic application yielded an estimate that the tubocurarine-receptor complex has a lifetime of about 2 sec (33), but those data were distorted by buffered diffusion within the synaptic cleft, and more recent experiments suggest a lifetime of several tens of milliseconds at the most (10, 29, 30). Further progress in understanding these lifetimes may come from experiments employing a "concentration jump" of antagonist during steady activation by agonists.

[2]Under some conditions tubocurarine acts in a more complicated fashion, probably because it also exerts "open-channel" blockade (29).

The photoisomerizable competitive antagonist, 2BQ, can be exploited for such experiments (Figure 1). Dose-response data show that the *cis* configuration binds about three times more tightly (dissociation constant, 1.5×10^{-7} M) than the *trans* isomer (66). The initial experiments with this compound utilized *cis* → *trans* photoisomerizations while channels were activated by photostable agonists such as carbachol and suberyldicholine; and the conductance increased, as expected (66). In all cases the conductance increase was dominated by a kinetic component with the rate constant of the agonist-receptor interaction (1–20 msec). Under some conditions there were slower components as well. These results indicate that 2BQ molecules can vacate receptors so rapidly (<0.5 msec) that agonist molecules can bind to receptors and open channels without a measureable delay. That is, antagonist-receptor kinetics are rapid on the time scale of channel gating. An ambiguity exists: Does this antagonist-receptor lifetime refer to the relatively potent *cis* configuration or to the looser (and presumably shorter-lived) *trans*-2BQ-receptor complex? These issues can be addressed with further experiments, especially ones involving *trans* → *cis* photoisomerizations, which have become possible recently because of improved light-flash technology.

MOLECULAR SCORECARD In general, the light-flash experiments with nicotinic acetylcholine receptors emphasize the rapid and tight coupling between events at ligand binding sites and events at the ion channel. Channel opening, closing, or blocking is detectable within a few microseconds of a flash-induced molecular rearrangement at the agonist binding site, or even of a "concentration jump" of ligands near receptors. This rapid temporal coupling probably reflects a close physical coupling among the polypeptide chains that comprise a single acetylcholine receptor monomer (48, 57, 115).

Granted that the light-flash relaxations *begin* within no detectable delay after the photochemical reaction. A more probing question is whether these relaxations *reach completion* with the ligand-receptor binding interaction itself or whether the kinetics are governed by slower processes such as conformational changes in the receptor protein (72, 73). Probably all investigators would now agree with the latter hypothesis if it were stated a bit more generally: Channel activation requires a further molecular event beyond the mere presence of two agonist molecules at their binding sites. The rate constant for the further transition, if it can be estimated from the leveling off in relaxation kinetics at high agonist concentration (91) or from relaxation kinetics with tethered QBr (65), is of the order of 1–3 $msec^{-1}$ at 12°C. According to this view, agonists (and antagonists, judging from the results with 2BQ) are equilibrating

with their binding sites on a more rapid time scale; how much more rapid is not yet certain, particularly since the light-flash studies have not yet revealed a signal associated with ligand binding. However the observed relaxation rates are already so fast that, for ligands at concentrations of 10^{-7} M such as Bis-Q and 2BQ, they make serious demands on the theory of encounter-limited chemical reactions. Perhaps the ligands in question are accumulating in high concentrations at the membrane-water interface (65, 108).

There are two possible exceptions to the picture that light-flash relaxations are dominated by intramolecular transitions rather than by binding and dissociation of agonists. (*a*) During phase 1, channels are closing so rapidly (>10 msec^{-1}, even at 10°C) that this may be the actual rate of *cis*-Bis-Q dissociation. However, this signal is too rapid for investigation with available voltage-clamp procedure. (*b*) Most investigators now feel that "open-channel blockade" occurs as the blocking molecule actually enters the channel and binds to a blocking site (2).

One may certainly anticipate further progress in understanding the link between ligand binding and channel behavior. Technical improvements are possible, and underway, in many of the kinetic experiments described here. However, it will be equally important to have more refined structural information on the receptor protein itself. This topic is under investigation in several laboratories.

Studies on Muscarinic Acetylcholine Receptors

In addition to their effects on nicotinic acetylcholine receptors, all the compounds shown in Figure 1 also interact with muscarinic acetylcholine receptors. At concentrations on the order of 10^{-5} M, they block the response to muscarinic agonists in frog heart. This action has been analyzed in detail using Bis-Q. In homogenates of frog heart, *trans*-Bis-Q blocks the binding of a radiolabeled muscarinic antagonist, [^{3}H]-*N*-methylscopolamine, with a dissociation constant of about 4 μM (79, 80). In voltage-clamped atrial trabeculae from frog heart, *trans*-Bis-Q inhibits the potassium conductance induced by muscarinic agonists. Dose-response curves for the agonist carbachol are shifted to the right, suggesting competitive blockade, with a dissociation constant of 5 μM. Both the binding and dose-response studies show that *cis*-Bis-Q is about 5-fold weaker as a muscarinic antagonist.

Light-flash experiments were conducted to measure the relaxations of potassium conductance in response to "concentration jumps" of a muscarinic antagonist (79, 80). As expected from the equilibrium data, *trans* → *cis* and *cis* → *trans* photoisomerizations led, respectively, to increases and decreases of the conductance. Also as expected from previous

studies using iontophoretic application of agonists, these relaxations contrasted in two ways with those seen at nicotinic receptors. First, they were much slower, requiring several seconds to reach completion. Second, they did not follow a simple exponential time course. Instead, both increases and decreases had an S-shaped waveform, including an initial delay or period of zero slope. The entire waveform of the relaxation, including the delay, was well described by the function $[1-\exp(-kt)]^2$, where t is the time since the flash. The rate constant k equaled 3 sec^{-1} at 24°C and had a Q_{10} of 2 to 2.5. However, within the range of conditions that allowed quantitative measurements, k did not depend on the nature of the agonist or on its concentration, on the concentration of Bis-Q, or on the membrane potential. These data indicate that the response to muscarinic agonists requires the sequential completion of at least two steps with roughly equal rates. The data provide little information on the molecular nature of these rate-limiting steps, except to rule out the agonist-receptor binding event. Many studies suggest that intracellular second messengers are involved in the response to muscarinic agonists; and therefore the experiments described in the next two sections may prove relevant for studies of muscarinic responses.

NUCLEOTIDES

Nucleotides have been generated by the photolysis of *o*-nitrobenzyl phosphate diesters and triesters (Figure 3).

Nucleoside Triphosphates

"Caged ATP" was originally synthesized and named by Kaplan et al (53). These workers showed that ATP is the only nucleotide formed upon irradiation. Thus, caged ATP is more useful than the α-unsubstituted analog; photolysis of the latter compound produces only 25% ATP, presumably because one of the photoproducts is an aldehyde that reacts with most of the ATP formed by photolysis (86).

Kaplan et al (53) showed that appreciable ATP could be generated from caged ATP after only 1 sec exposure to a mercury arc lamp. More detailed photochemical and information was obtained by McCray et al (75). Although the nitrobenzyl absorption peak is at 265 nm, the action spectum for ATP formation coincides with that for fluorescence excitation, with a peak at 315 nm. The efficiency of ATP formation per photon is decreased by only 50% at 347 nm, allowing for studies with a doubled ruby laser. This system gave 1% conversion per millijoule of flash, although absolute quantum yields were not measured. Frequency-doubled

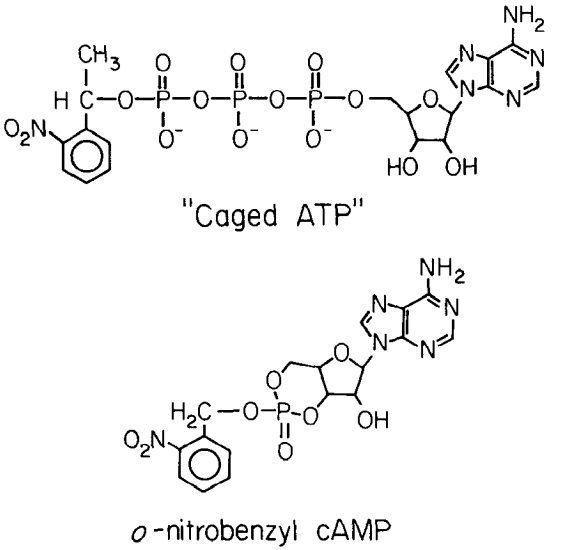


Figure 3 Photolabile esters of nucleotides.

ruby lasers can produce several tens of millijoules at this wavelength, thus affording substantial conversion.

McCray et al (75) also obtained kinetic information. Transient intermediates were followed directly with spectral monitoring; proton release was followed with pH indicators; and ATP formation was followed by measuring the turbidity decrease upon actomyosin dissociation. Within 5 μsec (the temporal resolution of the instruments) after the flash, a proton is released and an *aci*-nitro intermediate is formed. This intermediate decays into ATP in an acid-catalyzed step with a rate constant of 2.2×10^9 $[H^+]$ sec^{-1} at 22°C. Thus at pH 7, ATP is formed with a time constant of 4.5 msec. The reaction might be faster with suitable substituents on the benzene ring (112).

Caged ATP has desirable biochemical properties. The unphotolyzed molecule seems to be inactive toward ATPases such as the erythrocyte sodium pump (53), the calcium pump of the sarcoplasmic reticulum (47), actomyosin (49, 75), and bacterial nitrogenase (75). The only photoproducts are a proton, 2-nitrosacetophenone (which appears to have no harmful effects), and ATP itself. One can thus look forward to useful kinetic data in systems that are rate-limited by ATPases.

The same synthetic techniques would presumably yield photolyzable 2-nitrobenzyl esters of other mono-, di-, and triphosphonucleotides, as well as inorganic phosphate (46) and pyrophosphate. In particular,

"caged GTP" could become an important tool for exploring biological processes thought to be rate-limited by GTPases. Increased GTP hydrolysis accompanies hormonal stimulation of adenylate cyclase in many tissues (24, 55, 62, 68), and the GTPase step is thought to control the deactivation of adenylate cyclase (25, 26). There are also several analogies between this adenylate cyclase system and the regulation of a phosphodiesterase by a GTPase in rod outer segments (41, 69, 88).

Cyclic Nucleotides

Simultaneously with the development of caged ATP, Engels and his colleagues prepared 2-nitrobenzyl triesters of cyclic nucleotides. α-Unsubstituted esters were used and the aldehyde photobyproducts had no apparent toxic effects on the biological test systems. The 2-nitrobenzyl esters by themselves are not hydrolyzed appreciably by cyclic AMP-specific phosphodiesterase from beef heart; they are weak inhibitors of phosphodiesterase at millimolar concentrations (37, 58). At a concentration of 10^{-6} M, the *o*-nitrobenzyl triester of cAMP had less than 10% of the activity of cAMP itself in a protein kinase assay, but full activity was obtained after photolysis (36). These observations indicate that 2-nitrobenzyl triesters bind slightly, or not at all, to intracellular cyclic nucleotide receptors.

The triesters, if dissolved in 1% dimethyl sulfoxide and applied extracellularly, readily permeate cell membranes. Within a few minutes after exposure to the *o*-nitrobenzyl triester (100–500 μM), the physiological effects characteristic of cAMP begin to appear. These include morphological alterations in C6 glioma cells (37), positive inotroprotic effects in guinea pig heart (58), and spontaneous discharge in *Aplysia* bag cells (J. M. Nerbonne, L. K. Kaczmarek, and F. Strumwasser, unpublished). Evidently the cAMP is being generated in the cytosol from the *o*-nitrobenzyl triester. It is unclear whether spontaneous hydrolysis alone [half-time of 29 h at 35°C in physiological solution (58)] is rapid enough to account for this reaction; intracellular esterases might accelerate the breakdown of the triester.

It would be of great interest to monitor physiological responses while employing light flashes to accelerate (by many orders of magnitude) the appearance of cyclic nucleotides in the cytosol. The results with caged ATP suggest that such measurements are feasible, at least from the photochemical viewpoint. How rapidly will the "concentration-jump" of cAMP lead to activation of protein kinase, phosphorylation of proteins, and detectable physiological changes? In the heart, there is a lag of a few seconds between the stimulation of β-adrenergic receptors and the positive chronotropic effect (82, 89). Presumably some of this delay involves

the activation of adenylate cyclase and production of cAMP. The time course of subsequent events would be measured by the experiments suggested here.

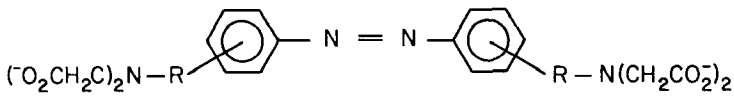
INORGANIC IONS

Calcium

Normal cells regulate the cytoplasmic concentration of calcium and hydrogen ions at about 10^{-7} M. Transient increases above this level occur when Ca^{2+} enters the cytoplasm either from the external solution or from an intracellular compartment. Increased Ca^{2+} levels activate muscle contraction, exocytosis, and several enzymes. The intracellular Ca^{2+} receptor is troponin C in the case of muscle contraction and the homologous protein, calmodulin, in many other cases.

In some cells, the intracellular [Ca^{2+}] increase produces a change in the ionic conductance of a membrane. A calcium-activated K^+ conductance was first noted in erythrocytes (114) and has now been described for neurons, cardiac muscle, salivary glands, and several other tissues (76). In recent experiments it was shown that Ca^{2+} is actually activating K^+ channels (71, 74). There remain several questions about the site of Ca^{2+} action, the kinetics of the membrane response, and the detailed relation between the local Ca^{2+} level and the membrane conductance. Such problems could be approached with a molecule that releases Ca^{2+} upon absorbing a photon.

A photosensitive chelator might be obtained by incorporating both (*a*) a photoisomerizable azobenzene group, to obtain large structural differences between *cis* and *trans* isomers and (*b*) several carboxylate groups, to obtain the Ca chelating activity of EDTA and EGTA (see also 103).



The compound should have the following characteristics:

1. One isomer must chelate Ca^{2+} with a dissociation constant not exceeding the intracellular Ca^{2+} concentration (10^{-7}–10^{-6} M). For the other isomer, the chelation must be much weaker—preferably by at least tenfold.
2. The compound must discriminate between Ca^{2+} and Mg^{2+}, with a strong preference for Ca^{2+} —preferably 1000-fold.

3. The compound must fulfill the usual requirements for thermal stability of the isomers, speed and efficiency of the photoisomerization, and lack of complicating pharmacology.

It is not yet known whether the desired characteristics can be achieved. As a first approach, the usual Ca^{2+} indicators were investigated for photolability. Like many azobenzene derivatives, the uncomplexed dye arsenazo III is transiently photochromic on a millisecond time scale (54a), presumably because the photo-generated *cis* configuration reisomerizes spontaneously with this time course. However it was not possible to demonstrate transient absorption changes or transient Ca^{2+} liberation from complexes of Ca^{2+} with arsenazo III, eriochrome black, or *o*-cresolphthalein complexone (A. Golob, H. A. Lester, unpublished). The azo bond itself may participate in the chelation and therefore be stabilized in the *trans* configuration. Similar negative results were obtained in attempts to bleach Ca^{2+}-murexide complexes.

In other initial investigations, Blank et al (21) have synthesized 4,4′-bis(α-iminodiacetic acid) azotoluene, in which R is a simple -CH_2-group *para* to the azo bond, and tested its ability to chelate Zn^{2+} ions. The compound is photoisomerizable and the *cis* configuration binds Zn^{2+} with an affinity constant of 1.1×10^5 M^{-1}, while binding to the *trans* configuration is much weaker. Neither isomer binds Ca^{2+} more tightly than about 10^3 M^{-1}, however (J. M. Nerbonne, unpublished results). In an effort to obtain a higher Ca affinity, the analog in which R is a *para*-ethoxy link was synthesized (Nerbonne, unpublished). This molecule binds Ca^{2+} with a dissociation constant of about 50 μM in the *trans* configuration and much more weakly in the *cis* configuration. Thus, progress is being made toward the goal of a light-sensitive Ca^{2+} chelator.

Protons

In comparison with Ca^{2+}, much less is known about the possible role of protons as an intracellular messenger. It is clear that many intracellular buffering processes exchange protons for Ca^{2+} ions—calmodulin and mitochondria are examples—so that any experimental strategy involving "Ca concentration jumps" must also control for, and examine, pH-jumps as well (77).

The difficulties are exemplified in current research on the control of some aspects of intercellular communication mediated by gap junctions. Pioneering studies in this field led to the conclusion that local increases in [Ca^{2+}] caused uncoupling (34, 70, 90). More recent work, however, has stressed the importance of hydrogen ions. Decreases of pH lead to the

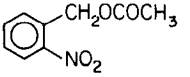
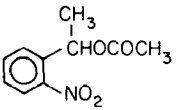
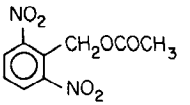
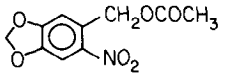
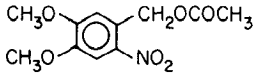
Structure	Name	λ_{max}, nm (ϵ, cm ℓ^{-1} mol^{-1})	λ_{irrad}, nm
	*o*-nitrobenzyl acetate	265 (4800)	300-366
	1-(*o*-nitrophenyl)-ethyl acetate	265 (4000)	300-366
	2,6-dinitrobenzyl acetate	280 (5200)	300-366
	6-nitropiperonyl acetate	355 (4200)	300-420
	3,4-dimethoxy-6 nitrobenzyl acetate	350 (4300)	300-420

Figure 4 Compounds synthesized for pH-jump experiments. The column labeled λ_{irrad} lists the lowest wavelength that we think will not damage cells and the highest wavelength where the compound absorbs appreciably.

disappearance of electrical coupling in amphibian and fish embryos (89a, 100, 104, 105) and in mouse pancreas (52). It would therefore be of interest to compare the time course of uncoupling after spatially and temporally defined increases of $[Ca^{2+}]$ and $[H^+]$.

With these points in mind, we have synthesized a series of nitrobenzyl esters that can be photolyzed to yield acetic acid and a (hopefully) inactive fragment (Figure 4). The unphotolyzed compounds are soluble in aqueous solution at concentrations up to 10^{-3} M and, being neutral, penetrate biological membranes without the need for micro-injection.

These compounds have been employed in preliminary intracellular "pH-jump" experiments with the gap junctions of *Chironomus* salivary glands (J. M. Nerbonne, unpublished). The pH is decreased by a 1 ms flash from a flash lamp. This leads to uncoupling on a time scale of seconds. Control experiments show that these effects are due to the pH jump rather than to the flashes alone or to the nitroso photoproducts. It is not yet known whether pH-jump relaxations also occur on the millisecond time scale that characterizes voltage-jump relaxations at gap junctions (45, 99).

GENERATION OF FLASHES

Flashlamp Systems

Commercially available flashlamp systems do not produce sufficient intensity for the experiments described in this review. Satisfactory custom-made systems are based on small "short-arc" flash tubes imaged with UV-efficient optics (79, 81). The flashes last about 0.5 msec.

Laser Systems

Several types of pulsed lasers produce flashes (duration < 1 μsec) suitable for the photochemical manipulations. Experiments with photoisomerizable azobenzene derivatives utilize a flashlamp-pumped dye laser optimized for 440 nm (59, 94, 95) or another one optimized for 350 nm (R. E. Sheridan and H. A. Lester, unpublished). Experiments on "caged ATP" were performed with a doubled ruby laser at 347 nm (75).

CONCLUSIONS

Pharmacological experiments with light flashes are now in various stages of refinement. Useful kinetic information has already been obtained in electrophysiological experiments with photoisomerizable cholinergic compounds. In other areas, such as "caged" nucleotides, the appropriate methods are available but are only now being systematically exploited. In other areas, such as Ca chelators, we probably still lack the necessary photosensitive compounds. Time will tell whether the experiments are focused on the right cells, the right molecules, and the right questions.

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